PYHIN genes as potential biomarkers for prognosis of human papillomavirus-positive or -negative head and neck squamous cell carcinomas.

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Abstract

The aim of the present study is to determine the expression levels of PYHIN (IFI16 and AIM2) and APOBEC3 (A3A, A3B, A3C, A3D, A3F, A3G, and A3H) gene family members in a cohort of patients with head and neck squamous cell carcinoma (HNSCC) and assess their potential correlation with human papillomavirus (HPV) infection status, clinical characteristics, and survival. For this purpose, 34 HNSCC tissue specimens along with healthy surrounding mucosa were collected from patients surgically treated for HNSCC. Nucleic acids were isolated to assess the presence of HPV and the expression levels of selected molecular markers. Survival analysis was carried out using the Kaplan-Meier method. In HPV-negative (HPV^-) HNSCCs, we detected low mRNA expression levels of IFI16, A3A, and A3B, whereas these genes were upregulated 2-100 folds in HPV-positive (HPV^+) tumors ($p<0.05$). Interestingly, AIM2 gene expression levels were predominantly unchanged in HPV^+ HNSCCs compared to their HPV^- counterparts, in which AIM2 was predominantly upregulated (10% *vs* 50% of patients). In HPV^- tumors, upregulation of TP53, NOTCH1, PD-L1, and IFI16 correlated with lower occurrence of nodal metastases. On the other hand, the expression of APOBEC family members did not correlate with clinical characteristics. Regarding survival, patients with upregulated A3F gene expression had a worse prognosis, while patients without changes in A3H expression had a lower survival rate. In conclusion, our findings indicate that the innate immune sensors IFI16 and AIM2 and some APOBEC family members could be potentially used as biomarkers for disease outcome in HNSCC patients regardless of HPV presence.

Key words: Head and neck cancer; Human papillomavirus; PYHIN proteins; APOBEC proteins; Survival

Introduction

Head and neck cancer is the sixth most common type of cancer and represents about 6% of all cases of tumors worldwide. Its incidence is 15.2 and 4.6 *per* 100,000 people in males and females, respectively [1]. Approximately 90% of all head and neck tumors, which arise predominantly in the mucosa of the oral cavity, pharynx, and larynx, are histologically classified as squamous cell carcinomas (HNSCCs).

Human papillomavirus (HPV), alongside other risk factors such as alcohol and tobacco consumption, is emerging as an important etiological factor of HNSCC. In this regard, HPV positivity correlated with an increasing incidence rate of oropharyngeal cancers in men younger than age 50 without a history of tobacco use, with HPV type 16 (HPV16) being the most commonly found high risk HPV in these HNSCCs [2]. Fittingly, high-risk HPV types have been detected in 45-70% of oropharyngeal cancers [3]. Intriguingly, different prognosis has been reported for and HPV-negative (HPV^-) *vs.* HPV-positive (HPV^+) HNSCCs. In particular, as HPV infection seems to be associated with a better response to therapy and survival [2], some authors have proposed a treatment deintensification for HPV^+ patients [4]. Despite significant progress in HNSCC disease management, the prognosis of recurrent HNSCCs still remains poor in most cases [5].

A number of HNSCC biomarkers have been identified over the last few years. These include oncogenes and tumor suppressors (e.g. TP53, p16, and NOTCH1), cell cycle regulators (e.g. Ki67, cyclin D1, EGFR, K-ras, pSTAT3, and SOX2), base excision repair pathway components (e.g. ERCC1, XRCC1), angiogenic factors (e.g. VEGF), and proteins involved in the immune response (e.g. PD-L1) [6]. Of note, TP53 and NOTCH1 appear to be the most mutated genes [6].

The observation that there are different biomarker profiles in HPV^- *vs.* HPV^+ HNSCCs [7] implies that HPV infection may be directly involved in gene regulation. In this regard,

HPV proteins E6 and E7 contribute to tumorigenesis through TP53 and retinoblastoma protein (pRb) inactivation. Loss of pRb activity induces p16 protein expression, regulating the progress from the G1 to the S phase of the cell cycle [7]. The immunohistochemical detection of p16 is used in routine diagnostics as a surrogate marker for HPV infection. However, its overexpression does not correlate univocally with the presence of HPV DNA in HNSCC, so it should not be considered as the ideal biomarker for HPV infection. Moreover, HPV^+ tumors express wild-type TP53 [8].

HPV^+ tumors display generally higher expression levels of interferon-inducible protein 16 (IFI16) compared to HPV^- lesions. IFI16, a member of the PYHIN family of proteins, [9][10]. In HNSCC, IFI16 exerts an *in vivo* anti-tumoral activity by promoting apoptosis of tumor cells, inhibiting neo-vascularization, and increasing the release of chemotactic factors for the recruitment of macrophages [11]. Furthermore, IFI16 has the ability to bind virus-derived intracellular DNA and then function as a pattern recognition receptor (PRR) [12,13,14].

Another member of the PYHIN family and cytoplasmic sensors of double strand DNA (dsDNA) of microbial or host origin is absent in melanoma 2 (AIM2), which plays an important role in the regulation of the inflammasome. Moreover, AIM2 orchestrates inflammasome-independent functions in colorectal cancer by suppressing stem cell proliferation [15].

In some human tumors, several mutations have been attributed to abnormal activity of DNA-modifying enzymes, including the apolipoprotein B mRNA editing enzyme catalytic polypeptide-like (APOBEC) family of cytidine deaminases [16,17,18,19]. APOBEC activation constitutes part of the innate immune response to viruses, including cytomegalovirus (CMV), HPV, and hepatitis B virus (HBV) [20,21,22]. APOBEC3A (A3A) and APOBEC3C (A3C) proteins can hypermutate the genome of HPV16 and reduce

pseudovirion infectivity [21]. Moreover, APOBEC3B (A3B) seems to play a major role in HPV^+ HNSCC mutagenesis [23]. Different mutational signatures have been found in HPV^- *vs.* HPV^+ HNSCC, with the former showing a smoking-associated mutational signature, while the reduced exposure to exogenous carcinogens in HPV^+ tumors creates a selective pressure that favors APOBEC-mediated mutations [23].

The primary aim of this study is to evaluate differences in gene expression levels between HPV^+ and HPV^- HNSCCs. For this purpose, we carried out gene expression analysis of a number of genes implicated in HPV-induced chronic inflammation and carcinogenesis such as the aforementioned innate immune sensors IFI16 and AIM2 and the APOBEC family members A3A, A3B, A3C, A3D, A3F, A3G, and A3H. Besides PYHIN and APOBEC genes, we assessed gene expression levels of TP53 and NOTCH1, two of the most altered genes in HNSCC, as well as that of MET, the receptor of hepatocyte growth factor (HGF) involved in the response to the anticancer agent cetuximab. Lastly, we measured mRNA levels of programmed death ligand 1 (PD-L1), a mediator of the immune response. The secondary aim is to determine any correlation between gene expression and clinical characteristics. Correlations with overall survival (OS) and disease-free survival (DFS) were analyzed as well.

Materials and methods

Patients and sample collection

One hundred patients surgically treated for HNSCC between January 2012 and September 2018 at Otorhinolaryngology Division of the University of Turin were enrolled in this study and evaluated for the presence of HPV. Exclusion criteria were the following: cancer of nasal cavities and nasopharynx, occult primary tumors, recurrent or second primary tumors, neoadjuvant chemotherapy, salvage surgery after radiation therapy or

chemoradiotherapy, tumor smaller than 1 cm in its maximum diameter (because the whole surgical sample was required for pathological examination), and distant metastases. The study sample included patients with carcinoma of oral cavity, oropharynx, larynx, and hypopharynx. HNSCC classification was based on the seventh edition of the American Joint Committee on Cancer (AJCC) staging system.

After surgical removal of the primary tumor, a portion of it (about 50-100 mg) and a sample of macroscopically healthy surrounding mucosa were isolated for molecular analyses. Patients underwent adjuvant treatments when adverse features were present at histology, according to national and international guidelines [5].

All procedures were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from each patient. This study was approved by the Research Ethics Committee of the University Hospital of Turin "A.O.U. Città della Salute e della Scienza di Torino–A.O. Ordine Mauriziano–A.S.L. Città di Torino" as well.

Ten HPV^+ HNSCCs (10% of all cases) were identified. They were analyzed for mRNA expression levels of selected biomarkers. Twenty-four consecutive HPV^- HNSCC patients were included in the study to assess the differences in mRNA expression levels. Therefore, molecular biomarkers were evaluated in a total of 34 HNSCC patients. Table 1 reports clinical characteristics of the two patient groups. Mean follow-up was 19.79 ± 8.05 months (range 7-36 months).

HPV detection

PCR analysis was carried out on tumor and mucosa DNA samples. The TRI Reagent® (Sigma-Aldrich, St. Louis, Missouri, USA) extraction kit was used for the simultaneous

extraction of DNA and RNA according to the manufacturer's protocol. DNA integrity was confirmed upon amplification of β-globin by PC04 and GH20 primers (Table 2). HPV DNA analysis was performed by nested PCR assay, using MY09-MY11 as the outer primers and GP5+-GP6+ as the inner primers (Table 2). The outer primer pair was specific for a conserved approximately 450 base-long sequence contained within the L1 gene, while the inner primers amplified a 150 base-long sequence within the sequence amplified by the first PCR assay [24,25]. All PCR reactions were carried out using REDTaq ReadyMix PCR Reaction Mix (Sigma, St. Louis, MO, USA) (Table 2). Finally, 15 µl of the PCR reaction mixtures were electrophoresed through a 1.8% agarose gel containing 0.5 µg/mL ethidium bromide and then visualized under an ultraviolet transilluminator.

RNA extraction and RT-qPCR analysis

To evaluate gene expression, RNA was extracted by TRI Reagent® (Sigma-Aldrich, St. Louis, Missouri, USA) extraction kit and 1 µg of RNA was retrotranscribed using the Revert-Aid H-Minus FirstStrand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA), according to the manufacturer's protocol. Quantification of mRNA expression in tumor and healthy mucosa samples was carried out using SYBR green-based RT-qPCR on a Mx3000P apparatus (Stratagene). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. Table 3 reports the primers used in RT-qPCR. The $2^{-\Delta\Delta Ct}$ method was applied to analyze the relative changes in gene expression from RT-qPCR experiments in the tumor tissue compared to the healthy mucosa from the same patient [26]. Gene expression classification was carried out according to Rusz et al. [27]. Briefly, a gene whose mRNA expression levels were found to be >2 times higher than those observed in surrounding healthy mucosa was classified as upregulated; if the values were <0.5 times or

between 0.5 and 2 times, the genes were classified as downregulated or unchanged, respectively.

Statistical analysis

All statistical analyses were carried out using Statistical Package for Social Sciences (SPSS), version 20.0, and GraphPad Prism, version 5. A descriptive analysis of all data was performed, and the data were reported as means or percentages and standard deviations. Since the Kolmogorov-Smirnov test demonstrated a non-Gaussian distribution of variables, nonparametric tests were used. The Mann-Whitney U test was employed to assess differences between groups in the mean of continuous variables. The chi-square (χ^2) or Fisher's exact test was used for categorical variables and the Kaplan Meier method for the creation of OS and DFS curves. Curve comparison was performed using the log-rank test. All tests were two-tailed. Adjustment for false discovery rate was used. A p value <0.05 was considered to be statistically significant.

Results

Patients and tumors characteristics

Table 1 shows the clinical characteristics of the HNSCC patients included in the study according to HPV status. Statistically significant differences were observed between the two groups in terms of alcohol consumption, smoke, and tumor site and stage (p<0.05 at χ^2 test, n = 34). In good agreement with the literature [2], the percentage of smokers and drinkers among HPV^+ patients was lower than that observed in HPV^- patients (30% *vs* 79% for smoke, and 20% *vs* 67% for alcohol consumption, for HPV^+ and HPV^- patients, respectively). The oropharynx was the main tumor site among HPV^+ patients (50% of cases), in good agreement with the notion that HPV affects this compartment more frequently than the oral cavity and

larynx. Finally, there were not stage IV tumors among HPV^+ patients probably because they had undergone exclusive chemoradiation therapy according to national and international guidelines [5].

Adjuvant treatment (i.e. radiotherapy or chemoradiotherapy) was administered in 50% and 40% of HPV^- and HPV^+ patients, respectively ($p>0.05$ at χ^2test, n = 34). Positive margins were found in 21% and 20% of HPV^- and HPV^+ patients, respectively ($p>0.05$ at χ^2 test, n = 34).

Gene expression of selected molecular markers correlates with HPV infection status

To determine potential correlations between HPV infection status and gene expression of selected biomarkers, we first measured mRNA expression levels by RT-qPCR in both the tumor tissue and the healthy mucosa from the same patient. Gene expression differences between these two compartments were then determined using the $2^{-\Delta\Delta Ct}$ method as described in the Materials and Methods. According to this method, genes were divided into three distinct groups: upregulated, downregulated, or unchanged. Fig. 1 shows gene expression levels according to the HPV status of the tumor. Statistically significant differences between HPV^- and HPV^+ HNSCCs ($p<0.05$ at χ^2test, n = 34) were observed for IFI16, AIM2, A3A, and A3B (Table 4, Fig. 1). Indeed, IFI16, A3A, and A3B were predominantly downregulated in HPV^- HNSCCs, while they were found upregulated in HPV^+ HNSCCs. This trend was particularly evident for IFI16 and A3B. Interestingly, AIM2 was four times more likely to be found as unchanged in HPV^+ HNSCCs compared to HPV^- HNSCCs, with a slightly higher percentage of these latter displaying AIM2 upregulation.

The other biomarkers did not show any significant differences ($p>0.05$ at χ^2test, n = 34). However, some genes displayed different expression patterns in HPV^- *vs.* HPV^+ HNSCCs. In particular, PD-L1 was more consistently found upregulated in HPV^+ *vs.* HPV^-

tumors. In contrast, A3H was predominantly downregulated in HPV^+ tumors. Finally, MET was among the most highly expressed genes in either group.

Relationships between gene expression and clinical characteristics

Next, we sought to determine any correlation between gene expression of selected biomarkers and the patients' clinical characteristics. To this end, we analyzed HPV^+ and HPV^- tumors separately to avoid a bias due to the fact that HPV infection correlated with tumor site and stage (Table 1). In HPV^- HNSCCs, a higher expression level of TP53, NOTCH1, and IFI16 correlated with a lower percentage of nodal metastases ($p<0.05$ χ^2 = 7.71, 6.62, and 6.17, respectively; n = 24) (Table 5, Fig. 2). Moreover, HPV^- patients without nodal metastases showed a higher expression of PD-L1 ($p<0.05$ χ^2 = 6.90; n = 24) (Table 5, Fig. 2). In HPV^- HNSCCs, higher TP53 expression levels were observed in early stage tumors (I-II) ($p<0.05$; χ^2 = 7.40; n = 24), whereas no correlation with tumor site, T status, or grading was observed. Moreover, gene expression of any APOBEC family members did not correlate with clinical characteristics. Lastly, in the HPV^+ group, there was not any statistically significant correlation with clinical characteristics ($p>0.05$ at χ^2test, n = 10).

Correlation among gene expression levels

We next asked whether there could be any correlation among the mRNA expression levels of APOBEC family members and the other genes so far examined. In HPV^- HNSCCs, we could find statistically significant correlations ($p<0.05$) between: 1) TP53 expression and A3A and A3F expression (χ^2 = 9.98 and 11.63, respectively; n = 24); 2) NOTCH1 expression and A3B and A3F expression (χ^2 = 12.73 and 12.14, respectively; n = 24); and 3) PD-L1 expression and A3A expression (χ^2 = 9.55; n = 24) (Table 6). In HPV^+ HNSCC, the following correlations ($p<0.05$) were observed between: 1) MET expression and A3A expression (χ^2 =

10.40; n = 10); 2) PD-L1 expression and A3F expression (χ^2 = 16.40; n = 10); and 3) IFI16 expression and A3A expression (χ^2 = 10.03; n = 10) (Table 6). In all cases, higher expression levels of the APOBEC gene were associated with higher expression levels of the other gene, indicating that these correlations were positive. After adjustment for false discovery rate, only the correlation between PD-L1 expression and A3F expression remained significant.

Survival analysis

Next, we sought to determine the prognostic value of the selected biomarkers. Firstly, we compared the OS and DFS of HPV^- HNSCC patients to those of HPV^+ HNSCC patients. In good agreement with the literature [2], we found a better OS and DFS for HPV^+ patients (Fig. 4). However, this trend was not statistically significant at log-rank test (p=0.418 for OS, and p=0.498 for DFS), probably because of the small number of patients and a mean follow-up < 3 years. Since the HPV^+ group comprised only a small number of patients (n=10), further survival analyses were performed only in the HPV^- group (Table 7).

According to previous results [5], patients without nodal metastases had a better OS and DFS (Fig. 5). Probably due to of the small sample size, this trend was not statistically significant at log-rank test (Table 7). Figures 6 and 7 show OS and DFS curves according to gene expression levels in the HPV^- group. Significance was only observed for A3F and A3H expression (Table 7). Interestingly, patients displaying upregulated A3F had a worse prognosis. A similar but not significant trend was observed for A3C. On the other hand, patients without changes in A3H expression had lower OS and DFS (Fig. 7). Lastly, patients with downregulated PD-L1, IFI16, and AIM2 seemed to have a worse prognosis (p>0.05), especially when considering OS (Fig. 6).

Discussion

HNSCC has an estimated incidence of about 16 new cases per 100,000 people, with a male to female ratio of approximately 2:1 [1]. According to the Surveillance, Epidemiology and End Results (SEER) database, there was an increase in incidence of oropharyngeal carcinomas from 1973 to 2001 in younger U.S. people (20-44 year-old), while the incidence of carcinomas in other oral and pharyngeal subsites remained constant [28]. This rise in the incidence of oropharyngeal carcinomas has been associated with the presence of HPV [29]. High-risk HPV types represent a risk factor for HNSCC independent of traditional risk factors such as tobacco and alcohol abuse. [30,31]

HPV^- and HPV^+ HNSCCs have different prognoses and molecular profiles [7]. In the last decades, a number of genes have been shown to display different mutation and gene expression patterns in HPV^- *vs* HPV^+ HNSCCs. For instance, TP53 mutations are the most frequent abnormalities found in HPV^- HNSCC [32,33]. Furthermore, TP53 mutations occur in 30-75% of HNSCCs and correlate with poor survival in invasive carcinomas [34,35,36]. The second most frequently mutated gene in HNSCC (14-15% of cases) is NOTCH1, a key player in normal cell differentiation, lineage commitment, and embryonic development. Enhanced expression levels of downstream NOTCH1 effectors have been found in 32% of HNSCCs [37], suggesting a pro-tumorigenic role of this gene in HNSCC pathogenesis [38]. In contrast, recent exome sequencing analyses have proposed a tumor suppressor role for NOTCH1 because of loss-of-function mutations found in a high percentage of patients [39]. Thus, further gene sequencing analyses will help clarify this important issue also in our cohort of patients.

Other mutated genes found in HNSCC, like MET, are also involved in the response to radiotherapy or chemotherapy and are known biomarkers of lymph node and distant metastases. Furthermore, MET and/or HGF are over-expressed in about 80% of HNSCC [37, 40, 41].

Finally, several mutated genes have been involved in the immunologic response to cancer. Among these, PD-L1 is a surface glycoprotein which induces T-cell anergy or apoptosis by binding to PD-1 on tumor infiltrating T lymphocytes [42]. PD-L1 overexpression has been observed in 61-71% of oropharyngeal carcinomas regardless of HPV status [43]. Inhibition of the PD-1:PD-L1 interaction with specific antibodies (i.e. immune-checkpoint inhibitors) is a promising antitumor treatment in patients with HNSCC, non-small cell lung cancer, and melanoma [43,44,45].

With regard to the aforementioned TP53, NOTCH1, MET, and PD-L1, our study does not show any statistically significant difference in gene expression between HPV^- and HPV^+ HNSCCs. Their expression patterns are, for the most part, in agreement with those described in the literature. MET was confirmed as the most frequently expressed gene, being detected in about 50-60% in our case series. In HPV^- tumors, TP53 and NOTCH1 negatively correlate with the presence of nodal metastases. In addition, TP53 appears to be negatively associated with tumor stage. These findings are consistent with the tumor suppressor role played by these genes in HNSCC pathogenesis [39]. A near significant direct association between MET and nodal metastases is in agreement with the literature [40]. In HPV^- tumors, PD-L1 showed a more complex relationship with nodal status. In fact, patients with nodal metastases had a lower percentage of tumors over-expressing PD-L1, in agreement with its role in evading immune response [42]. The remaining patients with nodal metastases mainly showed no changes in PD-L1 expression, thereby demonstrating a non-linear relationship. Thus, further studies on the complex interactions between tumor and immune system are clearly needed to better understand the clinical significance of our PD-L1 findings.

The role of PYHIN and APOBEC family member in HNSCC has only emerged in recent years [11,12,13,14,46]. In particular, co-expression of IFI16 and AIM2 was shown to enhance the cell growth in p53-deficient cells. By contrast, expression of IFI16 and/or AIM2

in cells with wild-type p53 suppresses the cell growth. Moreover, IFI16 and AIM2 synergistically enhanced NF-κB signaling in p53-deficient cells. Therefore, the expression of IFI16 and AIM2 appears to have oncogenic activities in tumor cells where p53 is inactivated [48]. However, the relationship between AIM2 expression and HPV infection has never been evaluated.

Here, we show a predominant upregulation of IFI16 in HPV^+ *vs* HPV^- tumors, in good agreement with the literature and consistent with its role as a viral restriction factor [47]. On the other hand, AIM2 was mainly upregulated in HPV^- HNSCC, while 50% of HPV^+ tumors showed no changes in AIM2 expression level. A previous study demonstrated aberrant expression of AIM2 in oral carcinomas, without looking at HPV infection status [48]. Since oral carcinomas rarely show HPV positivity, our results are likely to be consistent with the findings of the aforementioned study. The unexpected lack of overexpression of an intracellular sensor of viral DNA, like AIM2, in HPV^+ HNSCCs, which may relate to its predominant role in inflammasome regulation, warrants further investigation. Interestingly, IFI16 expression negatively correlated with nodal metastases in HPV^- HNSCCs, suggesting a protective role against the lymphatic diffusion of tumor cells also in the absence of HPV infection. The possible reason could be found in the regulation of some transcription factors, as demonstrated in differentiating B cells [49]. Our study seems to confirm a protective role for IFI16, in agreement with previous studies *in vitro* and *in vivo* [11,47], although further studies are required to further substantiate this hypothesis.

Finally, no significant correlation between IFI16 or AIM2 expression and clinical characteristics was observed in HPV^+ tumors. However, further studies with larger number of samples are clearly needed to generalize our findings.

Whereas the function of APOBEC deaminases in infection is well defined, their role in carcinogenesis has only recently emerged [16]. A3B is involved in HPV^+ HNSCC by

determining a peculiar mutational signature. In contrast, HPV^- tumors display a smoking-associated mutational signature. APOBEC activity induces helical domain hot spot mutations in the PIK3CA gene in HPV^+ HNSCC [23]. More recently, A3A has also been found overexpressed in HPV^+ oropharyngeal tumors [50]. Furthermore, enhanced A3A expression is associated with better overall survival in patients with oral cancer carrying A3B-deletion alleles [51].

Our study confirms the predominant role of overexpressed A3A and A3B in HPV^+ HNSCC. The percentages of patients characterized by overexpression of these two cytidine deaminases were 50% and 70%, respectively. The other APOBEC3 genes did not show any statistically significant difference in relation to HPV status, suggesting a weak role in head and neck carcinogenesis. None of the APOBEC3 genes showed any correlation with clinical characteristics (i.e. tumor site, T, N, stage, and grading) regardless of HPV status. However, the small number of patients in HPV^+ group may represent a limitation of our study. Only a few correlations between APOBEC3 expression levels and the other analyzed biomarkers were observed. These results are in line with the mutational activity of cytidine deaminases, which likely does not affect gene expression.

The survival analyses showed a better prognosis for HPV^+ HNSCC and for patients without nodal metastases (N0) , in agreement with the literature [2,5]. In this regard, the small sample size could represent a confounding factor that should be taken into account in subsequent survival analyses. When we evaluated patient survival in the HPV^- group, statistical significance for OS and DFS was achieved only for A3F and A3H genes. Specifically, patients characterized by A3F upregulation had a worse prognosis. On the other hand, patients without changes in A3H expression had lower OS and DFS. Our findings differ from those by Gao et al., showing a positive correlation between A3B, A3C, A3D, A3G, and A3H gene expression and survival in cervical cancer [52]. However, given that cervical

cancer is an HPV-related tumor and that our findings on A3F and A3H were obtained in HPV negative tumors, the disagreement between these two studies could be ascribed to the different HPV status of the tumors analyzed.

Of note, patients showing downregulation of PD-L1, IFI16, and AIM2 genes seemed to have a worse prognosis, especially in terms of OS. However, since patients without nodal metastases have a better prognosis, the correlation between nodal metastases and IFI16/PD-L1 could represent a confounding factor in such survival analyses.

With regard to TP53, since this gene is often mutated in HPV^- HNSCC, further studies taking into account both TP53 mutations and expression levels are clearly required to better understand its prognostic role. Furthermore, the upregulation of some APOBEC3 proteins in a percentage of HPV^- patients and their possible influence on survival should be further addressed in studies with a larger number of patients in order to better assess their prognostic value.

In conclusion, our findings are suggestive of a potential role of PYHIN and APOBEC family members as prognostic markers of HNSCC regardless of HPV infection status. Future studies are clearly needed to determine the predictive value of these biomarkers for response to surgery and chemoradiation therapy, which would then provide the rationale for HPV^+ patient treatment deintensification trials.

Compliance with Ethical Standards

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Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P Global cancer statistics, 2002. CA Cancer J Clin 55:74–108
2. Chaturvedi AK, Engels EA, Pfeiffer RM, et al (2011) Human Papillomavirus and Rising Oropharyngeal Cancer Incidence in the United States. J Clin Oncol 29:4294–4301. https://doi.org/10.1200/JCO.2011.36.4596
3. Hoffmann TK, Sonkoly E, Hauser U, et al (2008) Alterations in the p53 pathway and their association with radio- and chemosensitivity in head and neck squamous cell carcinoma. Oral Oncol 44:1100–1109. https://doi.org/10.1016/j.oraloncology.2008.02.006
4. Laskar S, Swain M (2015) HPV positive oropharyngeal cancer and treatment deintensification: How pertinent is it? J Cancer Res Ther 11:6. https://doi.org/10.4103/0973-1482.151445
5. Colevas AD, Yom SS, Pfister DG, et al (2018) NCCN Guidelines Insights: Head and Neck Cancers, Version 1.2018. J Natl Compr Cancer Netw 16:479–490.

https://doi.org/10.6004/jnccn.2018.0026

6. Lampri ES, Chondrogiannis G, Ioachim E, et al (2015) Biomarkers of head and neck cancer, tools or a gordian knot? Int J Clin Exp Med 8:10340–57
7. Thariat J, Vignot S, Lapierre A, et al (2015) Integrating genomics in head and neck cancer treatment: Promises and pitfalls. Crit Rev Oncol Hematol 95:397–406. https://doi.org/10.1016/j.critrevonc.2015.03.005
8. Cancer Genome Atlas Network (2015) Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature 517:576–582. https://doi.org/10.1038/nature14129
9. Goubau D, Rehwinkel J, Reis e Sousa C (2010) PYHIN proteins: center stage in DNA sensing. Nat Immunol 11:984–986. https://doi.org/10.1038/ni1110-984
10. Mondini M, Costa S, Sponza S, et al (2010) The interferon-inducible HIN-200 gene family in apoptosis and inflammation: implication for autoimmunity. Autoimmunity 43:226–31. https://doi.org/10.3109/08916930903510922
11. Mazibrada J, De Andrea M, Rittà M, et al (2010) In vivo growth inhibition of head and neck squamous cell carcinoma by the Interferon-inducible gene IFI16. Cancer Lett 287:33–43. https://doi.org/10.1016/j.canlet.2009.05.035
12. Unterholzner L, Keating SE, Baran M, et al (2010) IFI16 is an innate immune sensor for intracellular DNA. Nat Immunol 11:997–1004. https://doi.org/10.1038/ni.1932
13. Landolfo S, Gariglio M, Gribaudo G, Lembo D The Ifi 200 genes: an emerging family of IFN-inducible genes. Biochimie 80:721–8
14. Dell'Oste V, Gatti D, Giorgio AG, et al (2015) The interferon-inducible DNA-sensor protein IFI16: a key player in the antiviral response. New Microbiol 38:5–20
15. Man SM, Karki R, Kanneganti T-D (2016) AIM2 inflammasome in infection, cancer, and autoimmunity: Role in DNA sensing, inflammation, and innate immunity. Eur J

Immunol 46:269–80. https://doi.org/10.1002/eji.201545839

16. Kuong KJ, Loeb LA (2013) APOBEC3B mutagenesis in cancer. Nat Genet 45:964–5. https://doi.org/10.1038/ng.2736

17. Roberts SA, Lawrence MS, Klimczak LJ, et al (2013) An APOBEC cytidine deaminase mutagenesis pattern is widespread in human cancers. Nat Genet 45:970–6. https://doi.org/10.1038/ng.2702

18. Faden DL, Thomas S, Cantalupo PG, et al (2017) Multi-modality analysis supports APOBEC as a major source of mutations in head and neck squamous cell carcinoma. Oral Oncol 74:8–14. https://doi.org/10.1016/j.oraloncology.2017.09.002

19. Conticello SG (2008) The AID/APOBEC family of nucleic acid mutators. Genome Biol 9:229. https://doi.org/10.1186/gb-2008-9-6-229

20. Pautasso S, Galitska G, Dell'Oste V, et al (2018) Evasion Strategy of Human Cytomegalovirus to Escape Interferon-β-Induced APOBEC3G Editing Activity. J Virol JVI.01224-18. https://doi.org/10.1128/JVI.01224-18

21. Ahasan MM, Wakae K, Wang Z, et al (2015) APOBEC3A and 3C decrease human papillomavirus 16 pseudovirion infectivity. Biochem Biophys Res Commun 457:295–9. https://doi.org/10.1016/j.bbrc.2014.12.103

22. Lo Cigno I, De Andrea M, Borgogna C, et al (2015) The Nuclear DNA Sensor IFI16 Acts as a Restriction Factor for Human Papillomavirus Replication through Epigenetic Modifications of the Viral Promoters. J Virol 89:7506–20. https://doi.org/10.1128/JVI.00013-15

23. Henderson S, Chakravarthy A, Su X, et al (2014) APOBEC-mediated cytosine deamination links PIK3CA helical domain mutations to human papillomavirus-driven tumor development. Cell Rep 7:1833–41. https://doi.org/10.1016/j.celrep.2014.05.012

24. Fuessel Haws AL, He Q, Rady PL, et al (2004) Nested PCR with the PGMY09/11 and

GP5(+)/6(+) primer sets improves detection of HPV DNA in cervical samples. J Virol Methods 122:87–93. https://doi.org/10.1016/j.jviromet.2004.08.007

25. Rittà M, De Andrea M, Mondini M, et al (2009) Cell cycle and viral and immunologic profiles of head and neck squamous cell carcinoma as predictable variables of tumor progression. Head Neck. https://doi.org/10.1002/hed.20977

26. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–8. https://doi.org/10.1006/meth.2001.1262

27. Rusz O, Pál M, Szilágyi É, et al (2017) The Expression of Checkpoint and DNA Repair Genes in Head and Neck Cancer as Possible Predictive Factors. Pathol Oncol Res 23:253–264. https://doi.org/10.1007/s12253-016-0088-z

28. Shiboski CH, Schmidt BL, Jordan RCK (2005) Tongue and tonsil carcinoma. Cancer 103:1843–1849. https://doi.org/10.1002/cncr.20998

29. Hammarstedt L, Lindquist D, Dahlstrand H, et al (2006) Human papillomavirus as a risk factor for the increase in incidence of tonsillar cancer. Int J Cancer 119:2620–2623. https://doi.org/10.1002/ijc.22177

30. Dyson N, Howley PM, Münger K, Harlow E (1989) The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 243:934–7

31. Scheffner M, Werness BA, Huibregtse JM, et al (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63:1129–36

32. Westra WH, Taube JM, Poeta ML, et al (2008) Inverse relationship between human papillomavirus-16 infection and disruptive p53 gene mutations in squamous cell carcinoma of the head and neck. Clin Cancer Res 14:366–9. https://doi.org/10.1158/1078-0432.CCR-07-1402

33. Weinberger PM, Yu Z, Haffty BG, et al (2006) Molecular Classification Identifies a Subset of Human Papillomavirus–Associated Oropharyngeal Cancers With Favorable Prognosis. J Clin Oncol 24:736–747. https://doi.org/10.1200/JCO.2004.00.3335

34. Kumar B, Cordell KG, Lee JS, et al (2008) EGFR, p16, HPV Titer, Bcl-xL and p53, Sex, and Smoking As Indicators of Response to Therapy and Survival in Oropharyngeal Cancer. J Clin Oncol 26:3128–3137. https://doi.org/10.1200/JCO.2007.12.7662

35. Somers KD, Merrick MA, Lopez ME, et al (1992) Frequent p53 mutations in head and neck cancer. Cancer Res 52:5997–6000

36. Alsner J, Sørensen SB, Overgaard J (2001) TP53 mutation is related to poor prognosis after radiotherapy, but not surgery, in squamous cell carcinoma of the head and neck. Radiother Oncol 59:179–85

37. Kang H, Kiess A, Chung CH (2015) Emerging biomarkers in head and neck cancer in the era of genomics. Nat Rev Clin Oncol 12:11–26. https://doi.org/10.1038/nrclinonc.2014.192

38. Yap LF, Lee D, Khairuddin A, et al (2015) The opposing roles of NOTCH signalling in head and neck cancer: a mini review. Oral Dis 21:850–7. https://doi.org/10.1111/odi.12309

39. Agrawal N, Frederick MJ, Pickering CR, et al (2011) Exome Sequencing of Head and Neck Squamous Cell Carcinoma Reveals Inactivating Mutations in NOTCH1. Science (80-) 333:1154–1157. https://doi.org/10.1126/science.1206923

40. Ettl T, Viale-Bouroncle S, Hautmann MG, et al (2015) AKT and MET signalling mediates antiapoptotic radioresistance in head neck cancer cell lines. Oral Oncol 51:158–63. https://doi.org/10.1016/j.oraloncology.2014.11.005

41. Madoz-Gúrpide J, Zazo S, Chamizo C, et al (2015) Activation of MET pathway

predicts poor outcome to cetuximab in patients with recurrent or metastatic head and neck cancer. J Transl Med 13:282. https://doi.org/10.1186/s12967-015-0633-7

42. Flies DB, Sandler BJ, Sznol M, Chen L (2011) Blockade of the B7-H1/PD-1 pathway for cancer immunotherapy. Yale J Biol Med 84:409–21

43. Kim HS, Lee JY, Lim SH, et al (2016) Association between PD-L1 and HPV Status and the Prognostic Value of PD-L1 in Oropharyngeal Squamous Cell Carcinoma. Cancer Res Treat 48:527–536. https://doi.org/10.4143/crt.2015.249

44. Robert C, Schachter J, Long G V., et al (2015) Pembrolizumab versus Ipilimumab in Advanced Melanoma. N Engl J Med 372:2521–2532. https://doi.org/10.1056/NEJMoa1503093

45. Garon EB, Rizvi NA, Hui R, et al (2015) Pembrolizumab for the Treatment of Non–Small-Cell Lung Cancer. N Engl J Med 372:2018–2028. https://doi.org/10.1056/NEJMoa1501824

46. Bauml J, Seiwert TY, Pfister DG, et al (2017) Pembrolizumab for Platinum- and Cetuximab-Refractory Head and Neck Cancer: Results From a Single-Arm, Phase II Study. J Clin Oncol 35:1542–1549. https://doi.org/10.1200/JCO.2016.70.1524

47. Mazibrada J, Longo L, Vatrano S, et al (2014) Differential expression of HER2, STAT3, SOX2, IFI16 and cell cycle markers during HPV-related head and neck carcinogenesis. New Microbiol 37:129–43

48. Kondo Y, Nagai K, Nakahata S, et al (2012) Overexpression of the DNA sensor proteins, absent in melanoma 2 and interferon-inducible 16, contributes to tumorigenesis of oral squamous cell carcinoma with p53 inactivation. Cancer Sci 103:782–90. https://doi.org/10.1111/j.1349-7006.2012.02211.x

49. Piccaluga PP, Agostinelli C, Fuligni F, et al (2015) IFI16 Expression Is Related to Selected Transcription Factors during B-Cell Differentiation. J Immunol Res

2015:747645. https://doi.org/10.1155/2015/747645

50. Kondo S, Wakae K, Wakisaka N, et al (2017) APOBEC3A associates with human papillomavirus genome integration in oropharyngeal cancers. Oncogene 36:1687–1697. https://doi.org/10.1038/onc.2016.335

51. Chen T-W, Lee C-C, Liu H, et al (2017) APOBEC3A is an oral cancer prognostic biomarker in Taiwanese carriers of an APOBEC deletion polymorphism. Nat Commun 8:465. https://doi.org/10.1038/s41467-017-00493-9

52. Gao J, Choudhry H, Cao W (2018) Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like family genes activation and regulation during tumorigenesis. Cancer Sci 109:2375–2382. https://doi.org/10.1111/cas.13658

Figure legends

Fig. 1 Different patterns of gene expression in HPV$^-$ and HPV$^+$ HNSCCs. RNA samples from tumor tissues or healthy mucosa were subjected to RT-qPCR to measure gene expression levels of selected biomarkers. Values were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The HPV status was determined by nested PCR assays, using MY09-MY11 as the outer and GP5+-GP6+ as the inner primers on DNA obtained on the same samples (Table 2). Genes were classified as upregulated, downregulated or unchanged according to the differences in mRNA expression levels between the tumor and the surrounding healthy mucosa as described in the Materials and Methods. Asterisks refer to statistically significant differences between HPV$^-$ and HPV$^+$ HNSCCs (*$p<0.05$, chi-square test, A *vs* C, and B *vs* D).

Fig. 2 Gene expression of selected biomarkers in patients with or without nodal metastases. RNA samples from tumor tissues or healthy mucosa were subjected to RT-qPCR to measure gene expression of TP53 (A), NOTCH1 (B), PD-L1 (C), and IFI16 (D). Values

were normalized to GAPDH. Gene expression was classified as described in the legend to Fig. 1. N0 = patients without nodal metastases; N+ = patients with nodal metastases.

Fig. 3 Higher TP53 gene expression in early stages (I-II) of tumor progression. RNA samples from tumor tissues or healthy mucosa were subjected to RT-qPCR to measure TP53 mRNA expression levels. Values were normalized to GAPDH ($p<0.05$ at chi-square test, comparing early to late stages).

Fig. 4 Overall survival and disease free survival for HPV^- and HPV^+ HNSCC patients. (A) Overall survival (OS) was defined as the time from treatment to death due to any cause. (B) Disease free survival (DSF) was defined as the time from treatment to recurrence or death due to any cause. The Kaplan Meier method was employed for survival probability estimation.

Fig. 5 OS and DSF for HPV^- HNSCC patients with or without nodal metastases. OS (A) and DFS (B) were calculated as described in the legend to Fig. 4. The Kaplan Meier method was employed for survival probability estimation. N0 = patients without nodal metastases; N+ = patients with nodal metastases.

Fig. 6 Prognostic value of selected biomarkers in HPV^- HNSCCs. OS and DFS were calculated as described in the legend to Fig. 4. The Kaplan Meier method was employed for survival probability estimation. Gene expression analysis of the indicated biomarkers in HPV^- HNSCC patients was carried out as described in the legend to Fig. 1. (A) TP53, (B) NOTCH1, (C) MET, (D) PD-L1, (E) IFI16, (F) AIM2.

Fig. 7 Correlation between OS/DSF and APOBEC3 family members in HPV$^-$ HNSCCs. OS and DFS were calculated as described in the legend to Fig. 4. The Kaplan Meier method was employed for survival probability estimation. Gene expression analysis of the indicated APOBEC3 family members in HPV$^-$ HNSCC patients was carried out as described in the legend to Fig. 1. (A) A3A, (B) A3B, (C) A3C, (D) A3D, (E) A3F, (F) A3G, (G) A3H.

Table 1. Patients and tumors characteristics (number of patients, %).

Table 2. Primers and amplification protocols for HPV detection.

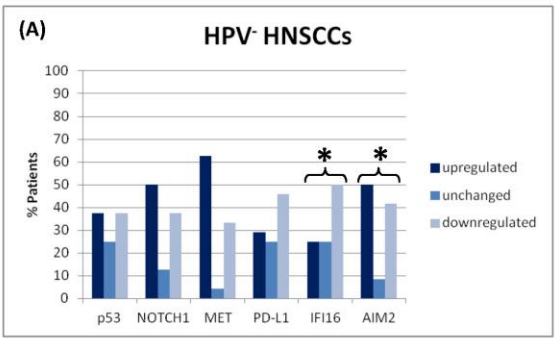
Table 3. Primers for RT-qPCR.

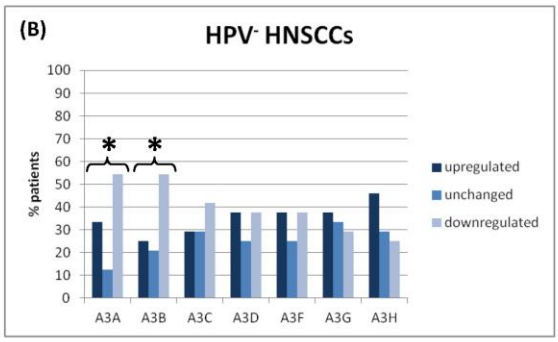
Table 4. Correlation between gene expression and HPV status (*p* value at chi-square or Fisher's exact test).

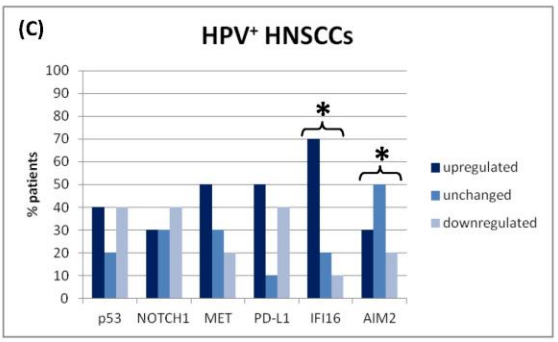
Table 5. Correlation between gene expression and clinical characteristics (*p* value at chi-square or Fisher's exact test) in HPV$^-$ and HPV$^+$ tumors.

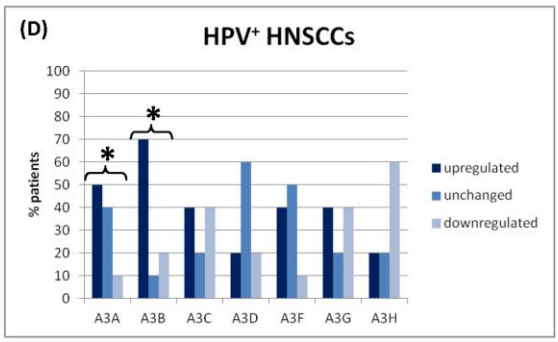
Table 6. Correlation among APOBECs expression and other genes (*p* value at chi-square or Fisher's exact test) in HPV$^-$ and HPV$^+$ tumors.

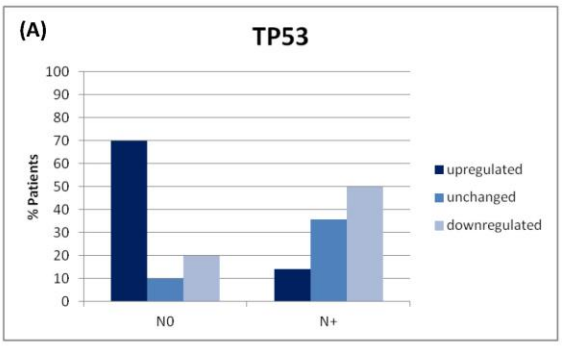
Table 7. *p* values at log-rank test for curve comparison (Overall Survival and Disease Free Survival) in HPV$^-$ patients.

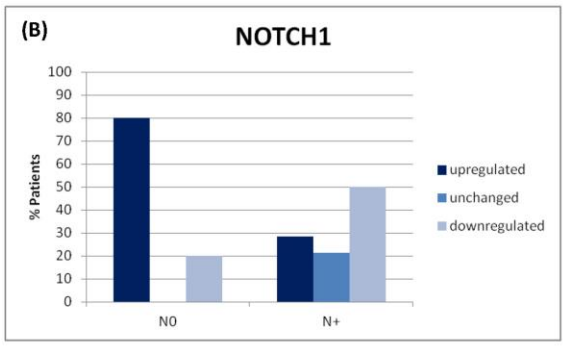

(A)
HPV- HNSCCs
% Patients
100
90
80
70
60
50
40
30
20
10
0
p53
NOTCH1
MET
PD-L1
IFI16
AIM2
upregulated
unchanged
downregulated

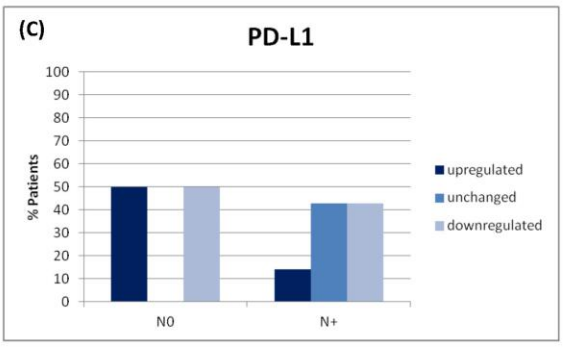

(B)
HPV- HNSCCs
% patients
100
90
80
70
60
50
40
30
20
10
0
A3A
A3B
A3C
A3D
A3F
A3G
A3H
upregulated
unchanged
downregulated

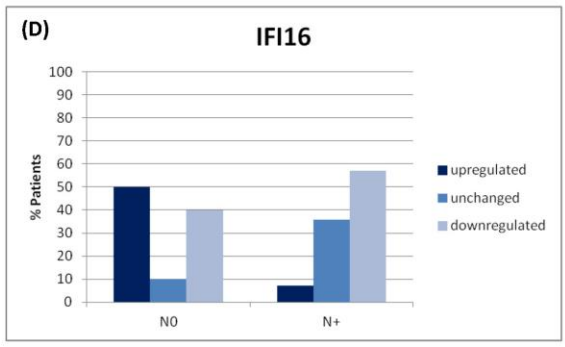

(C)
HPV+ HNSCCs
% patients
100
90
80
70
60
50
40
30
20
10
0
p53
NOTCH1
MET
PD-L1
IFI16
AIM2
upregulated
unchanged
downregulated

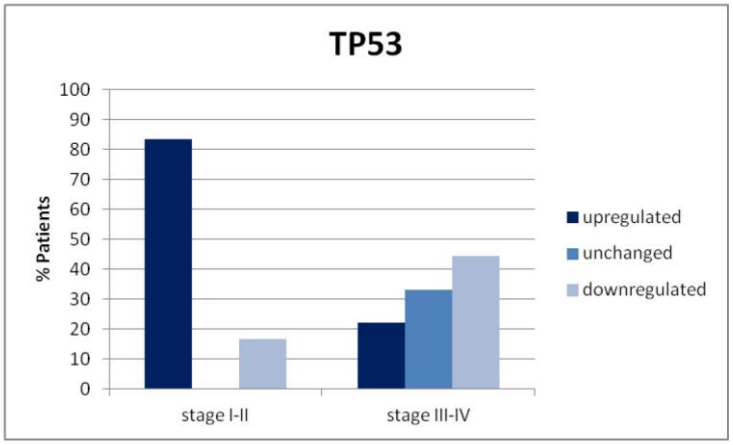

(D)
HPV+ HNSCCs
% patients
100
90
80
70
60
50
40
30
20
10
0
A3A
A3B
A3C
A3D
A3F
A3G
A3H
upregulated
unchanged
downregulated

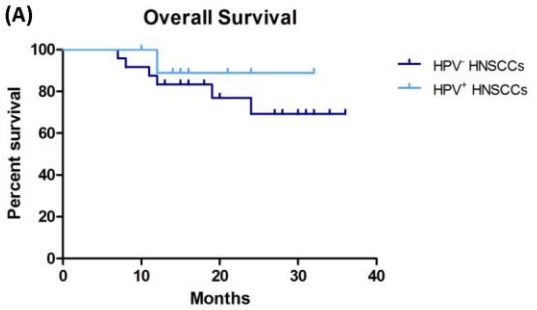

(A)
TP53
% Patients
100
90
80
70
60
50
40
30
20
10
0
N0
N+
upregulated
unchanged
downregulated

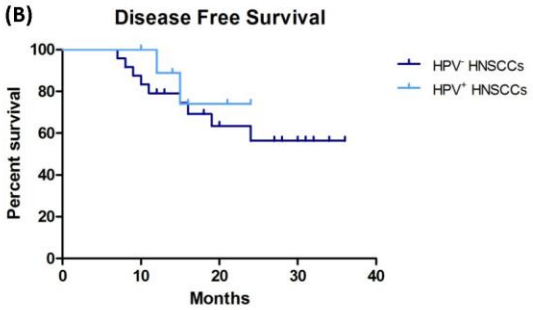

(B)
NOTCH1
% Patients
100
90
80
70
60
50
40
30
20
10
0
N0
N+
upregulated
unchanged
downregulated

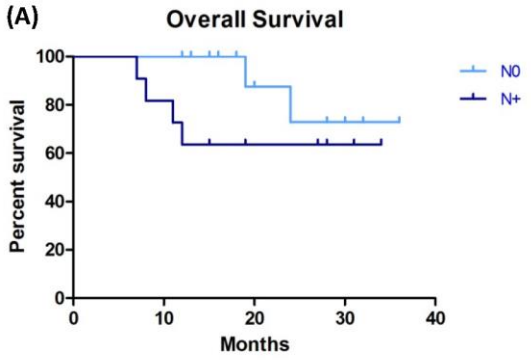

(C)
PD-L1
% Patients
100
90
80
70
60
50
40
30
20
10
0
N0
N+
upregulated
unchanged
downregulated

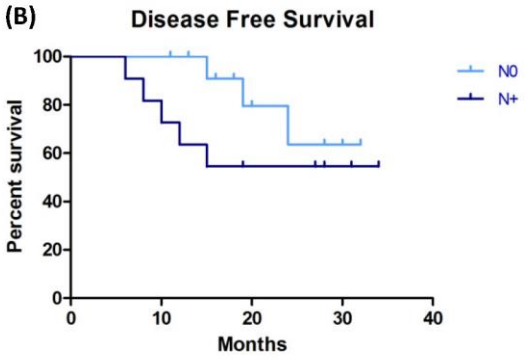

(D)
IFI16
% Patients
100
90
80
70
60
50
40
30
20
10
0
N0
N+
upregulated
unchanged
downregulated

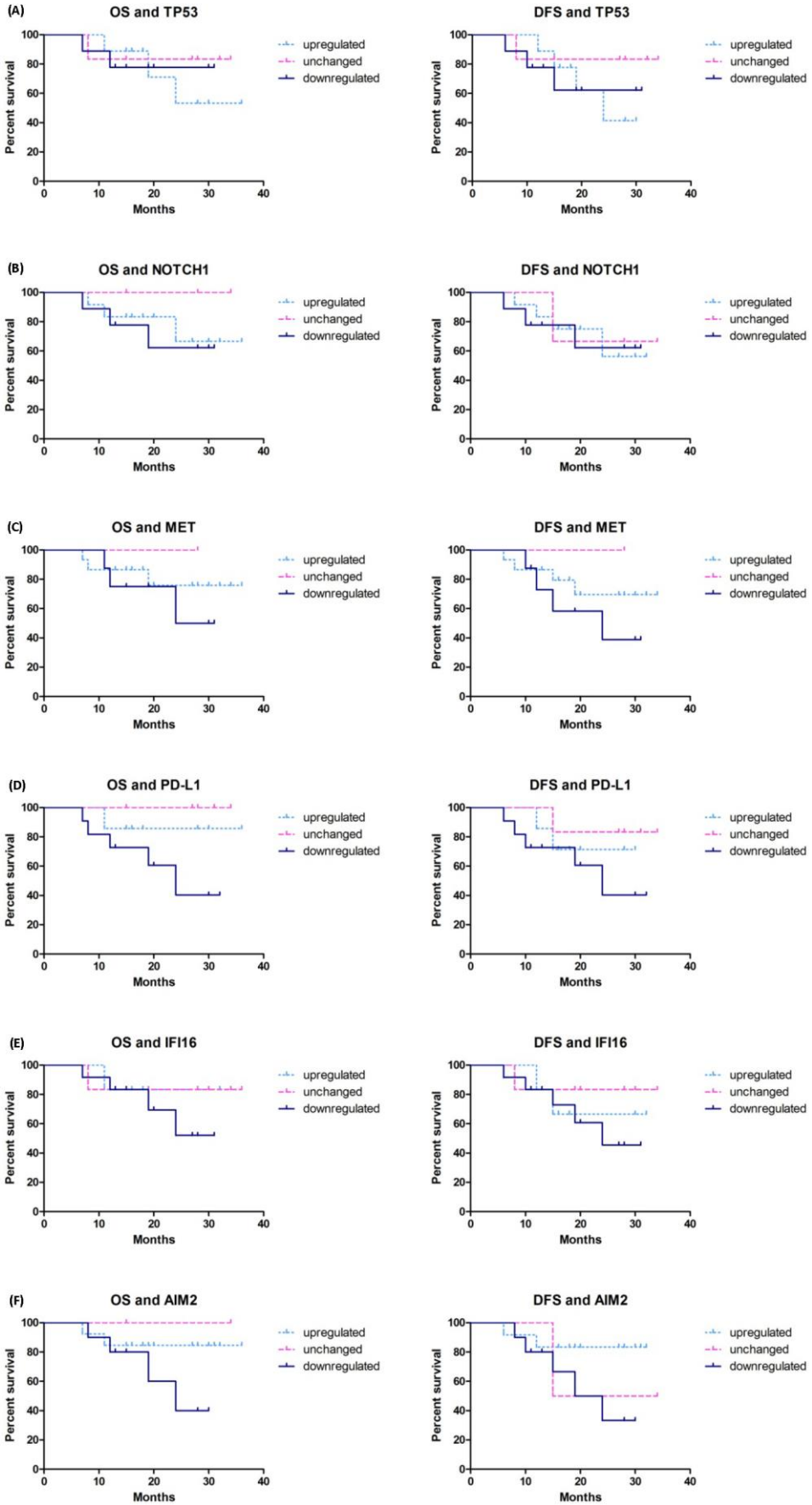

TP53
% Patients
100
90
80
70
60
50
40
30
20
10
0
stage I-II
stage III-IV
upregulated
unchanged
downregulated


(A)
Overall Survival
Percent survival
Months
HPV⁻ HNSCCs
HPV⁺ HNSCCs


(B)
Disease Free Survival
Percent survival
Months
HPV⁻ HNSCCs
HPV⁺ HNSCCs


(A)
Overall Survival
Percent survival
Months
N0
N+


(B)
Disease Free Survival
Percent survival
Months
N0
N+


(A)
OS and TP53
DFS and TP53
(B)
OS and NOTCH1
DFS and NOTCH1
(C)
OS and MET
DFS and MET
(D)
OS and PD-L1
DFS and PD-L1
(E)
OS and IFI16
DFS and IFI16
(F)
OS and AIM2
DFS and AIM2
upregulated
unchanged
downregulated
Percent survival
Months
0
10
20
30
40
0
20
40
60
80
100

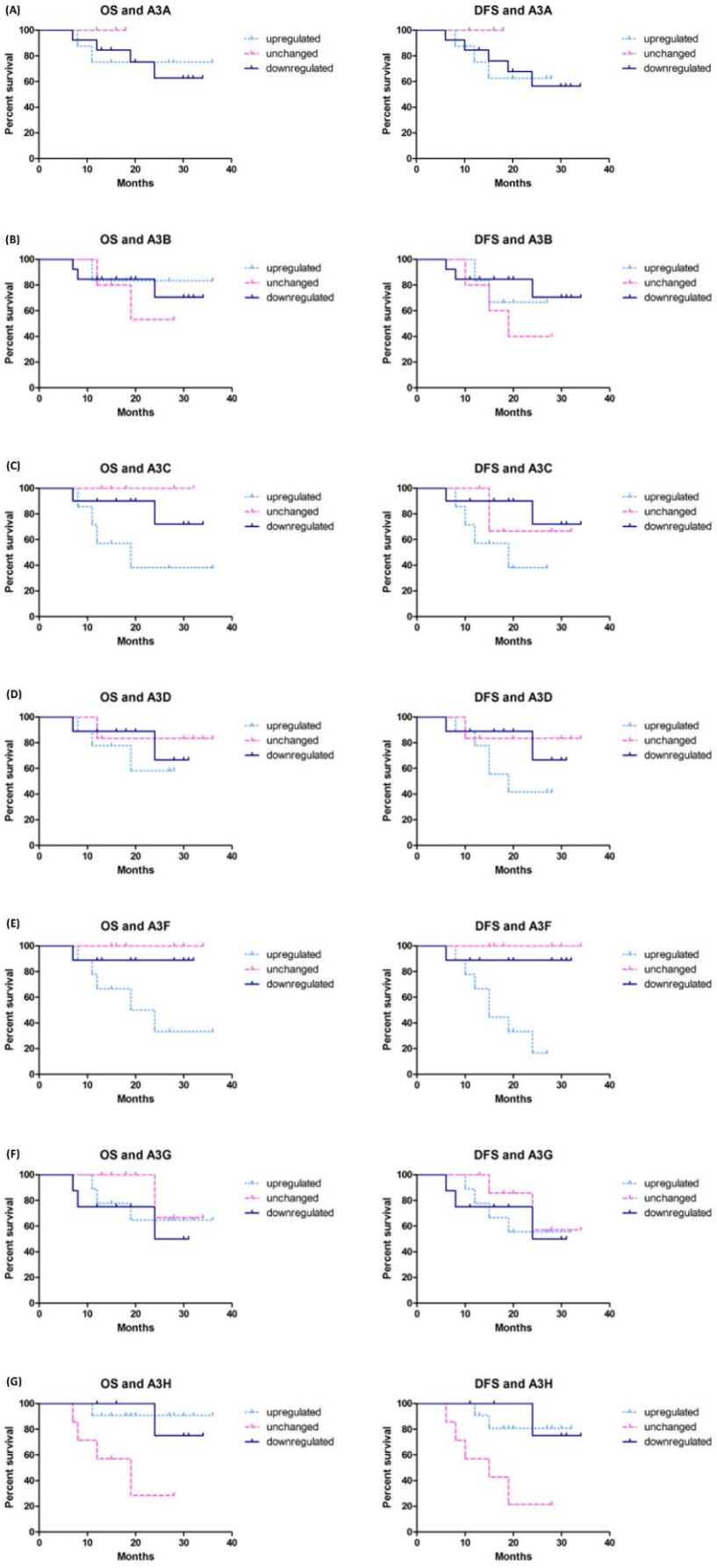

(A)
OS and A3A
DFS and A3A
(B)
OS and A3B
DFS and A3B
(C)
OS and A3C
DFS and A3C
(D)
OS and A3D
DFS and A3D
(E)
OS and A3F
DFS and A3F
(F)
OS and A3G
DFS and A3G
(G)
OS and A3H
DFS and A3H
upregulated
unchanged
downregulated
Percent survival
Months
0
10
20
30
40
0
20
40
60
80
100

Table 1. Patients and tumors characteristics (number of patients, %).

	HPV^-	HPV^+	*p* value
Age (mean ± st. dev.)	63.88 ± 13.20 years	60.90 ± 13.52 years	0.491
Sex			
Male	18 (75)	6 (60)	0.875
Female	6 (25)	4 (40)	
Alcohol consumption			
Yes	16 (67)	2 (20)	0.010
No	8 (33)	8 (80)	
Smoke			
Yes	19 (79)	3 (30)	0.009
No	5 (21)	7 (70)	
Site			
Oral cavity	15 (63)	4 (40)	0.005
Oropharynx	2 (8)	5 (50)	
Larynx	7 (29)	1 (10)	
Tumor (T)			
T1	6 (25)	2 (20)	0.279
T2	8 (33)	7 (70)	
T3	4 (17)	2 (20)	
T4a	6 (25)	0 (0)	
Nodes (N)			
N0	10 (42)	4 (40)	0.141
N1	9 (37)	4 (40)	
N2	5 (21)	2 (20)	
N3	0 (0)	0 (0)	
Metastases (M)			
M0	24 (100)	10 (100)	1.000
M1	0 (0)	0 (0)	
Stage			
I	5 (21)	0 (0)	0.017
II	3 (12)	4 (40)	
III	7 (29)	6 (60)	
IV A	9 (38)	0 (0)	
Grading			
G1	1 (4)	0 (0)	0.654
G2	11 (46)	6 (60)	
G3	12 (50)	4 (40)	

Table 2. Primers and amplification protocols for HPV detection.

	Primer sequences	Tm	Amplicon length
β-globin	PC04 (Fw): 5'-CAACTTCATCCACGTTCACC-3' GH20 (Rw): 5'-GAAGAGCCAAGGACAGGTAC-3'	55°C	268 bp
L1 outer primers	MY09 (Fw): 5'-CGTCCMARRGGAWACTGATC-3' MY11 (Rw): 5'-GCMCAGGGWCATAAYAATGG-3'	50°C	450 bp
L1 inner primers	GP5+ (Fw): 5'-TTTGTTACTGTGGTAGATACTAC-3' GP6+ (Rw): 5'-GAAAAATAAACTGTAAATCATATT-3'	56°C	150 bp

Tm = melting temperature

Table 3. Primers for RT-qPCR.

	Primer sequences	Tm	Amplicon length
GAPDH	Fw: 5'-AGTGGGTGTCGCTGTTGAAGT-3' Rw: 5'-AACGTGTCAGTGGTGGACCTG-3'	62°C	160 bp
TP53	Fw: 5'- CCCTTCCCAGAAAACCTACC-3' Rw: 5'-CTCCGTCATGTGCTGTGACT-3'	57°C	223 bp
NOTCH1	Fw: 5'-GGGTCCACCAGTTTGAATGG-3' Rw: 5'-GTTTGCTGGCTGCAGGTTCT-3'	58°C	306 bp
MET	Fw: 5'-AGCACTGCTTTAATAGGACAC-3' Rw: 5'-GATCGAGAAACCACAACCTG-3'	56°C	226 bp
PD-L1	Fw: 5'-CAATGTGACCAGCACACTGAGAA-3' Rw: 5'-GGCATAATAAGATGGCTCCCAGAA-3'	60°C	179 bp
IFI16	Fw: 5'-ACTGAGTACAACAAAGCCATTTGA-3' Rw: 5'-TTGTGACATTGTCCTGTCCCCAC-3'	59°C	432 bp
AIM2	Fw: 5'-AAGAAGGCAAGCAGGAGATG-3' Rw: 5'-GTTCAGCGGGACATTAACCT-3'	57°C	215 bp
APOBEC3A	Fw: 5'-GAGAAGGGACAAGCACATGG-3' Rw: 5'-TGGATCCATCAAGTGTCTGG-3'	56°C	61 bp
APOBEC3B	Fw: 5'-AATGTGTCTGGATCCATCAGG-3' Rw: 5'-TGAAGGTCAGCAATTCATGC-3'	56°C	105 bp
APOBEC3C	Fw: 5'-TCTGCATGACAATGGGTCTC-3' Rw: 5'-AAACTTGGCTGTGCTTCACC-3'	57°C	109 bp
APOBEC3D	Fw: 5'-GATCTGGAAGCGCCTGTTAG-3' Rw: 5'-AGTCGAATCACAGGCAGGAG-3'	58°C	110 bp
APOBEC3F	Fw: 5'-CCATAGGCTTTGCGTAGGTT-3' Rw: 5'-AATTATGCATTCCTGCACCG-3'	57°C	110 bp
APOBEC3G	Fw: 5'-TTCCAAAAGGGAATCACGTC-3' Rw: 5'-AGGGGCTTTCTATGCAACC-3'	56°C	95 bp
APOBEC3H	Fw: 5'-AGCTGTGGCCAGAAGCAC-3' Rw: 5'-CGGAATGTTTCGGCTGTT-3'	56°C	61 bp

Tm = melting temperature

Table 4. Correlation between gene expression and HPV status (*p* value at chi-square or Fisher's exact test).

	p value
TP53	0.952
NOTCH1	0.391
MET	0.099
PD-L1	0.429
IFI16	**0.034**
AIM2	**0.023**
A3A	**0.040**
A3B	**0.048**
A3C	0.785
A3D	0.151
A3F	0.203
A3G	0.707
A3H	0.142

Table 5. Correlation between gene expression and clinical characteristics (*p* value at chi-square or Fisher's exact test) in HPV^- and HPV^+ tumors.

HPV^- tumors

	TP53	NOTCH1	MET	PD-L1	IFI16	AIM2
Tumor site	0.479	0.496	0.616	0.737	0.392	0.872
T	0.468	0.740	0454	0.739	0.072	0.310
N	**0.021**	**0.036**	0.061	**0.032**	**0.046**	0.966
Stage	**0.025**	0.477	0.459	0.200	0.264	0.315
Grading	0.771	0.819	0.751	0.663	0.645	0.828

	A3A	A3B	A3C	A3D	A3F	A3G	A3H
Tumor site	0.726	0.880	0.948	0.075	0.385	0.742	0.594
T	0.480	0.353	0.116	0.055	0.930	0.235	0.800
N	0.408	0.537	0.550	0.226	0.796	0.212	0.105
Stage	0.604	0.243	0.358	0.227	0.862	0.568	0.762
Grading	0.789	0.323	0.097	0.566	0.220	0.318	0.521

HPV^+ tumors

	TP53	NOTCH1	MET	PD-L1	IFI16	AIM2
Tumor site	0.759	0.586	0.128	0.521	0.446	0.612
T	0.287	0.596	0.695	0.558	0.666	0.695
N	0.435	0.217	0.143	0.392	0.240	0.405
Stage	0.732	0.870	0.143	0.392	0.240	0.933
Grading	0.732	0.517	0.329	0.392	0.679	0.329

	A3A	A3B	A3C	A3D	A3F	A3G	A3H
Tumor site	0.116	0.833	0.123	0.240	0.298	0.759	0.558
T	0.558	0.582	0.155	0.349	0.255	0.287	0.596
N	0.082	0.679	0.153	0.108	0.287	0.732	0.435

Stage	0.392	0.679	0.435	0.435	0.392	0.732	0.435
Grading	0.659	0.240	0.054	0.108	0.170	0.732	0.870

Table 6. Correlation among APOBECs expression and other genes (*p* value at chi-square or Fisher's exact test) in HPV^- and HPV^+ tumors.

HPV^- tumors

	TP53	NOTCH1	MET	PD-L1	IFI16	AIM2
A3A	**0.041**	0.302	0.400	**0.049**	0.331	0.942
A3B	0.239	**0.013**	0.259	0.054	0.351	0.299
A3C	0.332	0.406	0.343	0.769	0.752	0.738
A3D	0.255	0.498	0.549	0.562	0.695	0.647
A3F	**0.020**	**0.016**	0.183	0.063	0.363	0.558
A3G	0.186	0.194	0.404	0.466	0.270	0.433
A3H	0.878	0.357	0.279	0.610	0.126	0.066

HPV^+ tumors

	TP53	NOTCH1	MET	PD-L1	IFI16	AIM2
A3A	0.579	0.622	**0.034**	0.541	**0.040**	0.231
A3B	0.169	0.513	0.546	0.351	0.277	0.403
A3C	0.870	0.472	0.530	0.251	0.392	0.287
A3D	0.645	0.191	0.695	0.558	0.666	0.109
A3F	0.759	0.092	0.612	**0.003**	0.369	0.107
A3G	0.181	0.057	0.123	0.263	0.104	0.123
A3H	0.155	0.349	0.155	0.558	0.582	0.695

Table 7. *p* values at log-rank test for curve comparison (Overall Survival and Disease Free Survival) in HPV^- patients.

	OS	DFS
N status (N0 vs N+)	0.202	0.220
TP53	0.793	0.598
NOTCH1	0.542	0.953
MET	0.609	0.470
PD-L1	0.099	0.348
IFI16	0.643	0.565
AIM2	0.298	0.247
A3A	0.735	0.674
A3B	0.787	0.518
A3C	0.052	0.210
A3D	0.636	0.273
A3F	**0.038**	**0.004**
A3G	0.551	0.776
A3H	**0.023**	**0.016**